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<b>(54) Title:</b> METHODS FOR THE PRODUCTION OF PROTEINS WITH A DESIRED FUNCTION		
<b>(57) Abstract</b> <p>The present invention provides a method for producing proteins with a desired function, generally comprising the steps of (a) providing a population of antibody-forming cells suspected of containing at least one cell capable of producing an antibody exhibiting a desired function; (b) suspending the population of antibody-forming cells in a medium, the medium having an indicator system incorporated therein, the indicator system also being capable of indicating the presence and location of a cell which forms antibodies exhibiting the desired function; (c) identifying a cell forming an antibody exhibiting the desired function; (d) isolating the identified antibody-forming cell from the medium; (e) determining the amino acid sequence of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell; and (f) synthesizing a protein with a desired function, the protein containing the amino acid sequence of the variable region or portion thereof which confers the desired function.</p>		

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<sup>+</sup> It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

## METHODS FOR THE PRODUCTION OF PROTEINS WITH A DESIRED FUNCTION

### Description

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#### Technical Field

The present invention relates generally to the production of proteins with a desired function, and more specifically, to methods for identifying specific antibodies or other proteins which can bind to a specific substance, and activate, catalyze, or otherwise effect a desired function. These methods also provide for modification of the identified protein, and for production of the protein in large quantities.

#### Background of the Invention

15 Proteins have long been recognized for their crucial role in many biological activities. Proteins in general have many capabilities, including, among others, the ability to activate or catalyze reactions, as well as the ability to cleave or modify other proteins, carbohydrates and organic compounds.

Although recombinant DNA techniques have been developed for producing useful proteins, one of the most difficult aspects of these processes lies in first detecting and isolating proteins of a desired biological activity. Once the protein has been detected and isolated in sufficient quantities, the structure of the gene encoding the protein may be deduced and used to produce the protein by recombinant DNA techniques. Various methods have been developed for detecting proteins of a desired biological activity, including a wide variety of biological assays. For example, proteins may be detected through the use of enzyme assays, binding assays which measure the affinity of proteins for specific substrates, or assays which detect the stimulation of cell growth.

One particular class of proteins, antibodies, has been identified and developed for therapeutic and diagnostic uses. Antibodies have a vast diversity of structure and function which allows virtually any given substance to be recognized. For example, antibodies may be found which are able to specifically bind to and/or neutralize particular toxins, viruses, parasites, and other etiologic agents. Additionally, antibody molecules have been identified that can mimic the functions of other proteins, such as growth factors, hormones, or enzymes. While methods have been developed to produce potentially unlimited quantities of specific monoclonal antibodies, these methods are severely limited in their

application due to the difficulty of isolating hybridomas from antibody-forming cells that occur only at low frequencies. More specifically, antibody-forming cells which produce antibodies with a restricted specificity, or with a particular functional activity such as the ability to mimic the function of an enzyme or hormone represent antibody-forming cells which are very difficult to isolate which are very difficult to isolate because they form only a small proportion of the antibodies that bind enzymes or hormone receptors. Moreover, some desired antibodies may be formed only by certain species of animals for which the hybridoma technology is unavailable.

Somatic cell hybridization is the most widely used method for producing monoclonal antibodies (see Kohler and Milstein, Nature 256:495, 1975). Although various modifications have been adopted in an effort to improve upon this method, such as *in vitro* immunization, electrofusion, and receptor-mediated strategies (see Monoclonal Antibody Production Techniques and Applications, Lawrence B. Schook (ed.), Marcel Dekker Inc., 1987), the method of somatic cell hybridization has remained essentially unchanged. Briefly, within this method, an animal is immunized with an antigen of interest inducing the development of an immune response by the animal to the antigen of interest. The animal is then sacrificed and organs with large deposits of B cells (such as the spleen and lymph nodes) are removed and fused to an immortal cell line, such as NS-1. The resulting hybridomas are then screened for specificity to the antigen of interest and subcloned until a single specific clone is obtained (see Paul G. Price, "Hybridoma Technology," Advances in Cell Culture, Vol. 4, 1985).

There are, however, many deficiencies with this method. One deficiency is that monoclonal antibodies cannot readily be made from human cells. The basic method requires immunization of an animal followed by the removal of its spleen and lymph nodes, which, of course, is not feasible for humans. Even if other techniques are used, such as *in vitro* immunization using human cells, there are other difficulties to overcome.

For example, it has proven difficult to find suitable human myeloma-fusion partners; human-human hybrids are not as stable and do not produce as great a quantity of antibody as can be attained in mouse-mouse fusion systems. Another difficulty is that human cells contain various repressed lethal viruses which may be activated and expressed upon hybridization and subsequent recombination. These viruses can be infectious, and pose issues of health and safety for lab workers. Furthermore, it is difficult to absolutely purify away all

lethal viruses from the monoclonal antibodies, and thus such antibodies could not readily be used therapeutically for humans.

Another difficulty of the basic method is obtaining rare antibodies. The size of the original pool of hybridomas is limited by the number of stable antibody clones that can be generated and screened in a reasonable time and by the intrinsic inefficiency of the process. Thus, of the antibody cells present in the population of immunized cells that are subjected to the fusion process, only a small fraction form stable antibody-producing hybrids and are available to screen for the desired antibody. Furthermore, antibodies must be subcloned in a tedious growth and subcloning process during which the desired antibody-forming cell may be lost. If the desired antibody is formed by only a small fraction of antibody-forming cells involved in an immune response, for example, an antibody which mimics an enzyme, the likelihood that this antibody will be produced by any of the stable hybrids available for screening is correspondingly small.

Yet another difficulty of the basic method is in varying the species of origin of the monoclonal antibody. While monoclonal antibodies can be produced from rodents, such as mice, rats and hamsters, they cannot readily be produced from other species, such as humans, as discussed above. Since the antibody repertoire differs for different animals, it would be advantageous if these intrinsic differences in repertoire could be exploited in order to produce a monoclonal antibody of a desired function or specificity.

Thus, there remains a need in the art for a method of producing proteins that replicate the binding characteristics and desired function of particular antibodies. The present invention fulfills this need, along with providing other related advantages.

#### Summary of the Invention

Briefly stated, the present invention provides a method for producing proteins with a desired function. In one aspect, the method comprises the steps of (a) providing a population of antibody-forming cells suspected of containing at least one cell capable of producing an antibody with a desired function; (b) suspending the population of antibody-forming cells in a medium, the medium having an indicator system incorporated therein, the indicator system also being capable of indicating the presence and location of a cell which forms antibodies exhibiting a desired function; (c) identifying a cell forming an antibody exhibiting the desired function; (d) isolating the identified antibody-forming cell from the medium; (e) determining the amino acid sequence of the variable region

or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell; and (f) synthesizing a protein with a desired function, the protein containing the amino acid sequence of the variable region or portion thereof conferring the desired function. Within an alternative embodiment, step (f) above is replaced with the following steps: (f) incorporating the DNA sequence corresponding to the amino acid sequence of the variable region or portion thereof conferring the desired function into a vector, the vector being capable of directing the expression and secretion of the protein with a desired function; (g) transfecting the vector into a host cell; (h) growing the vector into a host cell; and (i) isolating the protein with the desired function.

Within further embodiments, subsequent to the steps of isolating the antibody-forming cell, the method further comprises the steps of (a) recovering RNA from the isolated antibody-forming cell; (b) generating cDNA from the RNA; and (c) amplifying the cDNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell. As an alternative to steps (b) and (c), the following steps may be performed: (b) amplifying the RNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell; and (c) generating cDNA from the RNA. Within yet another alternative, steps (a), (b) and (c) are replaced with the following steps: (a) recovering DNA from the isolated antibody-forming cell; and (b) amplifying the DNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell.

Within yet another embodiment, subsequent to the step of determining the amino acid sequence, the amino acid sequence of the portion conferring the desired function is optimized such that the desired function is enhanced.

Within another aspect of the present invention, a method is provided for producing a protein with a desired function, comprising: (a) providing a population of antibody-forming cells suspected of containing at least one cell capable of producing an antibody exhibiting a desired function; (b) suspending the population of antibody-forming cells in a medium, the medium having an indicator system incorporated therein, the indicator system being capable of indicating the presence and location of a cell which forms antibodies exhibiting the desired function; (c) identifying a cell forming an antibody exhibiting the desired function; (d) isolating the identified antibody-forming cell

from the medium; (e) recovering RNA from the isolated antibody-forming cell; (f) generating cDNA from the RNA; (g) amplifying the cDNA of the portion of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell; (h) incorporating the amplified cDNA into a vector capable of directing the expression and secretion of a protein with the desired function; (i) transfecting the vector into a host cell; (j) growing the host cell in an appropriate medium; and (k) isolating the protein with the desired function from the host cell. Within a further embodiment of this invention, steps (f), (g) and (h) are replaced with the following steps:

10 (f) amplifying the RNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell; (g) generating cDNA from the RNA; and (h) incorporating the cDNA into a vector capable of directing the expression and secretion of a protein with the desired function.

15 In one embodiment of the present invention, the indicator system comprises a layer of cells whose growth viability or function is affected by antibodies exhibiting a desired function produced by the isolated antibody-forming cells. These antibodies have an effect analogous to a protein selected from the group consisting of IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IFN- $\alpha$ , IFN- $\gamma$ , G-CSF, M-CSF, GM-CSF, TNF- $\alpha$ , TGF- $\beta$ , erythropoietin, EGF, PDGF, and the ligand of the kit protein. In another embodiment, the indicator system comprises one or more pathogenic microorganisms, and a layer of cells susceptible to infection by the microorganisms, wherein the antibodies exhibiting a desired function are identified as those which affect the pathogenicity of the

20 microorganism. In another embodiment, the layer of cells susceptible to infection by the microorganisms is adhered to a glass slide or petri dish. In yet another embodiment, the indicator system comprises a set of two cell types selected from the group consisting of cells of distinct HLA histocompatibility antigen types, blood group antigen types, or tumor cells and normal cells of the same lineage. In yet another embodiment, the indicator system comprises erythrocytes or other particles coated with antigen, and the antibodies of a desired function are identified as those which bind to the coated antigen, causing the particles to agglutinate.

25 In another embodiment of the present invention, the indicator system comprises erythrocytes coated with antigen such that the antibodies of a desired function are identified as those which bind to the coated antigen and lyse cells in the presence of complement. Anti-immunoglobulin antibodies may be

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added such that complement fixation is enhanced. In another embodiment, the indicator system comprises a substrate, and the antibody exhibiting a desired function is identified as that which modifies the substrate in a detectable manner. In yet another embodiment of the invention, the indicator system comprises a  
5 complexing factor, and the antibody-forming cell is identified as that which binds to the complexing factor, forming a rosette. Suitable complexing factors include erythrocytes bound to an antigen, particles bound to an antigen, and microorganisms.

Also provided by the present invention are proteins which are  
10 produced by the above described methods.

These and other aspects of the present invention will become evident upon reference to the following detailed description.

#### Detailed Description of the Invention

15 Prior to setting forth the invention, it may be helpful to an understanding thereof to define certain terms to be used hereinafter.

Indicator System: An indicator system manifests a specific desired activity in response to antibody on the surface of or released into the vicinity of an  
20 antibody-forming cell, thus permitting identification and isolation of the cell-producing antibody with the desired function.

Antibody-Forming Cell: A cell which forms antibodies, such as a B-lymphocyte or its progeny including the plasma cell. These cells may either  
25 secrete antibodies (antibody-secreting cells) or maintain antibodies on the surface of the cell without secretion into the cellular environment. Other antibody-forming cells within the scope of the present invention include bacterial cells, mammalian cells, yeast or other cells which form antibodies as a result of the artificial expression of introduced immunoglobulin genes or parts thereof  
30 according to the general methods described by Winter and colleagues (see E. S. Ward et al., Nature 341:544, 1989; see also R. Orlandi et al., Proc. Natl. Sci. USA 86:3833, 1989) and by Lerner and colleagues (see W. D. Huse et al., "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275, 1989).



**Medium:** A substance that provides the minimum requirements for the short-term maintenance of cellular integrity and cellular structures. These requirements may be met by, for example, an isotonic buffer.

5           **Nutrient Medium:** A substance that contains carbon and nitrogen sources as well as other supplements which are required for the viability of cultured cells. Such media may be obtained from commercial sources or formulated from published recipes. (See catalogs of the American Type Culture Collection, Rockville, Md.)

10           As noted above, the present invention simplifies the mass production of proteins having a desired function. These proteins have the same binding characteristics as the antibody produced by a single carefully selected, antibody-forming cell and possess a biological activity which allows them to bind, catalyze, activate, or otherwise effect a desired function. The present invention  
15 allows the rapid identification and exploitation of such antibody-forming cells, even if present at only a very low frequency.

          An antibody-forming cell may be identified among antibody-forming cells obtained from an animal which has either been immunized with a selected substance, or which has developed an immune response to an antigen as a result  
20 of disease. Animals may be immunized with a selected antigen using any of the techniques well known in the art suitable for generating an immune response (see HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, D.M. Weir (ed.), Vol 4, Blackwell Scientific Publishers, Oxford, England, 1986). Within the context of the present invention, the phrase "selected antigen" includes any substance to which an  
25 antibody may be made, including, among others, proteins, carbohydrates, inorganic or organic molecules, and transition state analogs that resemble intermediates in an enzymatic process. Suitable antigens include, among others, biologically active proteins, hormones, cytokines, and their cell surface receptors, bacterial or parasitic cell membrane or purified components thereof, and viral  
30 antigens. Representative cytokines include IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IFN- $\alpha$ , IFN- $\gamma$ , G-CSF, M-CSF, GM-CSF, TNF- $\alpha$ , TGF- $\beta$ , erythropoietin, EGF, PDGF, and the ligand of the kit protein. Representative viral antigens include reverse transcriptase core and envelope proteins, for example, gp 120, the envelope protein of HIV-1. As will be appreciated by one of ordinary skill in the  
35 art, antigens which are of low immunogenicity may be accompanied with an adjuvant or apten in order to increase the immune response (for example,

complete or incomplete Freund's adjuvant) or with a carrier such as keyhole limpet hemocyanin (KLH).

Many warm-blooded animals, such as humans, rabbits, mice, rats, sheep, cows or pigs may be immunized in order to obtain antibody-forming cells. However, mice and rats are generally preferred because of their ease in handling, well-defined genetic traits, and the fact that they may be readily sacrificed. Procedures for immunizing animals are well known in the art. Briefly, animals are injected with the selected antigen against which it is desired to raise antibodies. The selected antigen may be accompanied by an adjuvant or hapten, as discussed above, in order to further increase the immune response. Usually the substance is injected into the peritoneal cavity, beneath the skin, or into the muscles or bloodstream. The injection is repeated at varying intervals and the immune response is usually monitored by detecting antibodies in the serum using an appropriate assay that detects the properties of the desired antibody. Large numbers of antibody-forming cells can be found in the spleen and lymph node of the immunized animal. Thus, once an immune response has been generated, the animal is sacrificed, the spleen and lymph nodes are removed, and a single cell suspension is prepared using techniques well known in the art.

Antibody-forming cells may also be obtained from a subject which has generated the cells during the course of a selected disease. For instance, antibody-forming cells from a human with a disease of unknown cause, such as rheumatoid arthritis, may be obtained and used in an effort to identify antibodies which have an effect on the disease process or which may lead to identification of an etiological agent or body component that is involved in the cause of the disease. Similarly, antibody-forming cells may be obtained from subjects with disease due to known etiological agents such as malaria or AIDS. These antibody-forming cells may be derived from the blood or lymph nodes, as well as from other diseased or normal tissues. Antibody-forming cells may be prepared from blood collected with an anticoagulant such as heparin or EDTA. The antibody-forming cells may be further separated from erythrocytes and polymorphs using standard procedures such as centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Antibody-forming cells may also be prepared from solid tissues such as lymph nodes or tumors by dissociation with enzymes such as collagenase and trypsin in the presence of EDTA.

Antibody-forming cells may also be obtained by culture techniques such as *in vitro* immunization. Examples of such methods are described by C. R. Reading in Methods in Enzymology 121:18-33 (J. J. Langone, H. H. van Vunakis

(eds.), Academic Press Inc., N. Y.). Briefly, a source of antibody-forming cells, such as a suspension of spleen or lymph node cells, or peripheral blood mononuclear cells are cultured in medium such as RPMI 1640 with 10% fetal bovine serum and a source of the substance against which it is desired to develop  
5 antibodies. This medium may be additionally supplemented with amounts of substances known to enhance antibody-forming cell activation and proliferation such as lipopolysaccharide or its derivatives or other bacterial adjuvants or cytokines such as IL-1, IL-2, IL-4, IL-5, IL-6, GM-CSF, and IFN- $\gamma$ . To enhance immunogenicity, the selected antigen may be coupled to the surface of cells, for  
10 example, spleen cells, by conventional techniques such as the use of biotin/avidin as described below.

Antibody-forming cells may also be obtained from very early monoclonal or oligoclonal fusion cultures produced by conventional hybridoma technology. The present invention is advantageous in that it allows rapid selection  
15 of antibody-forming cells from unstable, interspecies hybridomas, e.g., formed by fusing antibody-forming cells from animals such as rabbits, humans, cows, pigs, cats, and dogs with a murine myeloma such NS-1.

Antibody-forming cells may be enriched by methods based upon the size or density of the antibody-forming cells relative to other cells. An example of  
20 the use of Percoll to separate cells according to density is described by van Mourik and W. P. Zeizlmaier in Methods in Enzymology 121:174-182 (J. J. Langone, H. H. van Vunakis (eds.), Academic Press Inc., N.Y.). Gradients of varying density of solutions of bovine serum albumin can also be used to separate cells according to density. (See N. Moav and T. N. Harris, J. Immunol. 105:1512, 1970;  
25 see also Raid, D. J. in SELECTED METHODS IN CELLULAR IMMUNOLOGY, B. Mishell and S. Shiigi (eds.), W. H. Freeman and Co., San Francisco, 1987). The fraction that is most enriched for desired antibody-forming cells can be determined in a preliminary procedure using the appropriate indicator system (as described below) in order to establish the antibody-forming cells.

30 In order to identify single antibody-forming cells producing antibodies of a desired function, the cells suspected of possessing the desired function are suspended in a medium having an "indicator system" incorporated therein. Within the context of the present invention, "having an indicator system incorporated therein" includes not only indicator systems which are located within  
35 the medium containing the antibody-forming cells, but indicator systems which are positioned contiguous to the medium, such that the effects of the antibodies produced by the antibody-forming cells may be manifested in the indicator system.

Additionally, the effects of the antibodies may be transferred to the indicator system by other means. For example, a membrane may be positioned contiguous to the medium in order to adsorb antibodies which diffuse from the antibody-forming cells. Suitable membranes include nitrocellulose or other materials which bind proteins. To further enhance local adsorption of the antibodies, the membrane may be coated with proteins such as an antibody or antibodies which is specific for immunoglobulins of the same species as that of the antibody-forming cell. The membrane acts as a replica upon which the distribution of antibody-forming cells in the media may be reproduced. When the membrane is placed into one of the indicator systems described below, antibody-forming cells producing antibodies of a desired function may be detected on the membrane. An antibody-forming cell which forms a positive in the indicator system may be located by aligning the membrane with the gelified media. If the antibody-forming cells are bacterial cells, the colonies or plaques may similarly be replicated by plaque or colony lifts using methods known to those skilled in the art.

It will be evident to those skilled in the art that in addition to establishing the presence of cell-producing antibodies of a desired function within a population of cells, the indicator system may also be utilized to optimize procedures for immunization regimens, to determine sources of antibody-forming cells which produce antibodies which confer the desired function, or to determine the efficacy of protocols for enriching antibody-forming cells.

The medium incorporating the indicator system should at a minimum provide the requirements for the short-term maintenance of cellular integrity and structures. The medium may be in the form of a liquid layer, or a gelified layer. Methods for gelifying media are well known in the art. Particularly preferred gelified media contain about 0.3%-0.6% agar or about 1.0% methyl cellulose. Adherence of gelified media to glass may be facilitated by precoating the glass. For example, glass slides may be brushed with a hot solution of 0.1% (w/v) agarose and allowed to dry, followed by addition of a gelified medium. If a viable cell is required after isolation of the antibody-forming cell, then it is preferred to use a nutrient medium with appropriate supplements in order to aid cell viability. Particularly preferred media is RPMI 1640 (Gibco, Grand Island, N.Y.), with 10% Fetal Bovine Serum (Hyclone Serum) and 20 mM HEPES (Gibco). Within certain embodiments of the present invention, the indicator cells, and in some cases the antibody-forming cells, may be adhered to the surface of a microscope slide or dish culture. Adherence may be induced by coating the

surface of the slide or culture dish with poly-L-lysine (Sigma Chemical Company, Mo.) or fibronectin (Sigma Chemical Co.). Within one embodiment, 1 mg/ml of poly-L-lysine, or 100 µg/ml of fibronectin is used to coat a slide or culture dish surface for about one hour, followed by rinsing with Phosphate Buffered Saline (PBS).

The components of the indicator system are selected so as to manifest activity on the surface of an antibody-forming cell. Additionally, in cases where antibody is secreted from the surface of the antibody-forming cell, antibody will diffuse into the nearby vicinity of the cell. Thus, in indicator systems which produce plaques (as discussed below), diffusion of antibody into the surrounding indicator system results in a plaque with an individual antibody-forming cell located centrally therein. Antibody-forming cells should be diluted within the indicator system to a density which allows selection of an individual or small number of antibody-forming cells. If it is unclear which cell is responsible for the activity indicated by the indicator system, or in order to confirm the activity, the selected cell(s) may be retested for their ability to produce antibodies with the desired effect by transfer to a fresh indicator system.

Through use of the methods described herein, the desired function of the antibody identified by the indicator system may be replicated in another antibody or other protein, allowing production of the antibody or protein with the desired function in virtually unlimited quantities. By this means a variety of useful proteins with desired functions may be produced, including proteins with the function of mimicking the activity of biologically active factors, such as IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IFN-α, IFN-γ, G-CSF, M-CSF, GM-CSF, TNF-α, TGF-β, erythropoietin, EGF, PDGF, the ligand of the kit protein, or of other biologically active substances. The present invention also allows the production of proteins with a function analogous to antibodies produced only in human beings, such as antibodies that neutralize a microorganism that is pathogenic only in humans (e.g., the HIV-1 virus). Such proteins may be used in the passive immunotherapy of viral diseases, such as AIDS, or in identifying important vaccine candidates among antigens of a microorganism including, among others, viruses, parasites, yeast, and bacteria. The proteins could also be used to discriminate between human histocompatibility antigens, blood group antigens, or to bind to autoantigens and aid in their characterization. The proteins can also be used as catalysts to carry out enzymatic reactions such as the cleavage of proteins.

In one aspect of the present invention, the indicator system comprises a layer of cells whose growth, viability, or function is affected by antibodies produced by the isolated antibody-forming cell. For example, to identify an antibody-forming cell which produces an antibody that neutralizes a growth factor or other biologically active molecule, the indicator system contains indicator cells which respond to the presence of the biologically active molecule. The biologically active molecule may cause cells to survive rather than die, to change morphology, or to spread and adhere to a surface. Interference with the action of the biologically active factor in the vicinity of the antibody-forming cell indicates the presence of an antibody which neutralizes the growth factor or biologically active molecule.

In contrast, the indicator system may be prepared with a layer of cells, excluding a necessary biologically active molecule. With the exclusion of the biologically active molecule, antibodies that are able to mimic the particular action of the biologically active molecule and reproduce the desired change in the indicator cell, e.g., by influencing viability, morphology, adherence, or other properties, may be detected. Antibodies so detected may exhibit biological activity analogous to a protein such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IFN- $\alpha$ , IFN- $\gamma$ , G-CSF, M-CSF, GM-CSF, TNF- $\alpha$ , TGF- $\beta$ , erythropoietin, EGF, PDGF or the ligand of the kit protein.

Within another aspect of the present invention, antibodies may be identified which neutralize the pathogenicity of a microorganism. Within the context of the present invention, microorganisms include viruses, parasites, bacteria, and yeasts or fungi. To identify antibodies neutralizing the pathogenicity of a microorganism, susceptible cells and the microorganism are mixed in the indicator system with a source of antibody-forming cells. Desired antibodies are identified by the lack of a cytopathic effect on susceptible cells in the region of the antibody-forming cell. If the indicator cells are adherent, they may be allowed to adhere to glass slides or petri dish before the gelified medium containing antibody-forming cells is added. As noted above, if the indicator cells are nonadherent, poly-L-lysine or fibronectin may be used to coat the surfaces and thus aid the adherence of these cells. Within a preferred embodiment, a suspension of the indicator cells in medium is applied to the surface at a concentration of approximately  $2 \times 10^6$  cells/cm<sup>2</sup> and allowed to stand for thirty minutes to an hour at 37°C. After the cells have adhered the surplus medium may be removed.



Examples of such indicator systems include baby rabbit kidney (BRK) cells and vaccinia virus, or human T-lymphocyte cell lines and HIV-1, any of which may be obtained from conventional sources such as the American Type Culture Collection (Rockville, Md.). Antibody-forming cells which release an antibody that neutralizes infection by the virus are identified by their central position in a plaque of indicator cells that have been protected from infection and have not undergone a cytopathic effect.

Within one embodiment of the present invention, a Balb/C mouse is immunized with an inactivated preparation of vaccinia virus. After several immunizations, the mouse is sacrificed and the spleen and lymph nodes are removed. The spleen and lymph nodes are disassociated to form a single-cell suspension of antibody-forming cells, and are mixed together with baby rabbit kidney cells and allowed to adhere to poly-L-lysine treated glass or plastic. After incubating at 37°C in nutrient medium to allow recovery of metabolic activity of the antibody-forming cells, the nutrient medium is removed and replaced with a thin layer of nutrient medium containing 0.5% methyl cellulose together with a titre of vaccinia virus shown in pilot experiments to be just sufficient to produce confluent lysis of the baby rabbit kidney cells over a 24-hour period. The indicator system is incubated in a highly humidified CO<sub>2</sub> incubator for 24 hours. Antibody-forming cells that produce antibody which neutralizes the virus result in plaques of live baby rabbit kidney cells (which may be identified with phase contrast microscopy) surrounding a centrally positioned antibody-forming cell.

Within another aspect of the present invention, the indicator system comprises a mixture of two or more cell types. Examples include cells of distinct HLA histocompatibility antigen types, different blood group antigen types, and tumor cells and normal cells of the same lineage. The two types of cells should be distinguishable by morphology, or by marking of one of the populations by for example, a fluorescent marker. Antibodies produced by the antibody-forming cell are then selected based upon their ability to agglutinate or lyse one cell of the pair.

Within one embodiment, the indicator system is utilized to detect a cell producing an antibody that discriminates between a tumor cell and a normal cell of the same cell lineage or tissue type as the tumor cell. This may be accomplished by the desired antibody agglutinating or including complement mediated lysis of the tumor cells, but not the normal cells in the vicinity of the antibody-secreting cell. If the tumor cell and the normal cell cannot be readily distinguished by morphology, they may be distinguished by labeling one of the pair

with a dye, for example, with a fluorescent dye, such as fluorescein isothiocyanate. Cells may be readily coupled with this dye by incubation with 100 µg/ml of fluorescein isothiocyanate (Molecular Probes, Plano, Tex.) in phosphate-buffered saline at pH 8 for thirty minutes. The cells are then washed with phosphate-buffered saline and 10% fetal bovine serum. Cells may also be stained with a colored substance such as trinitro-phenol (TNP) using 2-4-6 trinitro-phenol benzene sulphonic acid by exposing them to a solution of 10 mg/ml in 0.28 cacodylate buffer, pH 6.9 for 30 minutes at room temperature (SELECTED METHODS IN CELLULAR IMMUNOLOGY, B. Mishell and S. Shiigi (eds.), W. H. Freeman and Co., San Francisco, 1987, pp. 112-113).

Within a preferred embodiment, antibody forming cells at concentrations ranging from  $10^3$  to  $10^5$  cells/ml are mixed together with tumor and nontumor cells at a concentration of  $10^6$  cells/ml. The mixture is supplemented with a source of complement, such as guinea pig serum that has been absorbed with sheep erythrocytes, at a dilution such as 1 in 16 by final volume, together with an enhancing anti-immunoglobulin serum. The enhancing anti-immunoglobulin serum is generally prepared utilizing either a rabbit or goat which has been immunized and has developed antibodies against the immunoglobulins of the animal species from which antibody-forming cells were derived. This serum is diluted as determined by pretrial experiments, generally in the range of 1 in 80 to 1 in 400 by final volume. Antibody-forming cells which generate an antibody that binds only to tumor cells, will result in a plaque of tumor cells, leaving only nontumor cells surrounding a centrally located antibody-forming cell. Phase contrast microscopy with or without the addition of a dye such as trypan blue may be used to identify plaques or regions where cells have been lysed by the action of antibodies, the enhancing anti-immunoglobulin antibodies, and complement.

Within another aspect of the present invention, the indicator system comprises erythrocytes or other particles coated with antigen, and the antibody exhibiting a desired function is identified as that which binds to the antigen, thereby causing the particles to agglutinate or lyse in the region surrounding the antibody-forming cell. Many particles may be used in the context of the present invention, including, among others, sheep erythrocytes and artificial particles, such as beads (Polysciences, Warrington, Penn.) or magnetic beads (Robbin Scientific, Mountain View, Calif.). A number of methods for coating particles with antigens are known to those skilled in the art. These include the use of chromic chloride



(see C. R. Parish and J. A. Hayward, Proc. Roy. Soc. 187:47, 1974) or water-soluble carbodiimide (see E. S. Golub et al., J. Immunol. 100:133, 1968).

In a preferred embodiment, a biotin/avidin coupling system is utilized for plaque or "rosetting" assays in order to detect antibodies that bind to a particular antigen. Briefly, cells (for example, sheep red blood cells) are washed thoroughly by centrifugation with phosphate-buffered saline (PBS). Half a ml of packed red blood cells is then suspended in 5 ml of PBS which has been adjusted to pH 8-8.3 by the addition of sodium hydroxide. A succinimide ester derivative of biotin dissolved in dimethyl sulfoxide is added to the PBS, and the mixture is agitated at room temperature for about one hour. The red cells are then washed with PBS, pH 7, and may be stored in the refrigerator for up to two weeks.

The selected antigen which is to be coupled to the red cells is dialyzed overnight in 0.1 M NaHCO<sub>3</sub> at a concentration of 1 mg/ml. The succinimide ester of biotin is dissolved in dimethyl sulfoxide (DMSO) and added to the solution of the protein, and the mixture is held at room temperature for one hour. The biotin-conjugated protein is then dialyzed overnight against PBS with 0.1% sodium azide and stored by refrigeration.

To conjugate biotin-coupled proteins to the red blood cells, 0.2 ml of packed biotin-conjugated red cells are first incubated at room temperature with an excess of avidin (2 ml of 1 mg/ml avidin in PBS, pH 7). The red cells are separated from the avidin solution by centrifugation and are washed with PBS, pH 7. The avidin-coated red cells are then added to a solution of the biotin-coupled protein and the mixture held at room temperature for one hour. The red cells are then separated from the solution by centrifugation and washed three times in PBS, pH 7.

The optimal conditions for coating the red cells with individual proteins may be determined empirically in the chosen indicator system by varying the concentration of biotinylated protein or the degree of biotinylation of the protein. Coating of red cells with biotin may be monitored using fluorescein-conjugated avidin and fluorescence microscopy or flow cytometry. Likewise, successful coating of the red cells with the protein may be assessed with a fluorescein-conjugated antibody directed against that protein.

The antigen-coated red cells are preferably used in an assay based upon the ability of specific antibodies, through complement-mediated lysis, to produce a plaque of hemolysed red cells around an antibody-forming cell secreting the desired antibody. If agar is used to gelify the medium, anti-complementary activity should be neutralized by inclusion of 1 mg/ml DEAE

dextran, Mr  $5 \times 10^5$  to  $2 \times 10^6$  (Pharmacia Fine Chemicals). To enhance complement-mediated hemolysis of antigen-coated erythrocytes by the desired antibodies, anti-immunoglobulin antibodies or "enhancing" antisera may be included in the indicator system. This antisera or antibody is specific for immunoglobulins of all classes or of a desired class of immunoglobulin in the species from which the antibody-forming cells are derived, and is able to fix complement when bound to antibody molecules bound to the antigen-coated erythrocytes. Within one embodiment, enhancing antisera derived from a sheep immunized with murine immunoglobulins is utilized to enhance hemolysis of murine antibodies. The optimal concentration of enhancing antisera may be determined by observing the concentration enabling detection of the greatest number of hemolytic plaques in a plaque assay. Within this embodiment the indicator system contains a source of antibody-forming cells (e.g., nucleated spleen cells from a mouse immunized with an antigen, against which it is desired to produce an antibody), antigen-coated sheep erythrocytes (e.g., at  $5 \times 10^8$ /ml), a source of complement, (e.g., guinea pig serum preabsorbed with two equal volumes of sheep erythrocytes at a final concentration of 10% by volume), and enhancing serum prepared as described above at a final concentration of 0.5%, all in nutrient medium buffered with 20 mM HEPES. The mixture is introduced dropwise under a layer of warm (37°C) paraffin oil on a transparent surface, e.g., a glass slide, and incubated for one hour.

Antibody-forming cells which produce an antibody which binds to the antigen coated on the sheep erythrocytes may be identified by their central position in plaques of hemolysed red blood cells with an inverted phase-contrast microscope. To exclude the possibility that the antibody-forming cell is producing an antibody that is not specific for the antigen which is coupled to the erythrocyte, but rather the biotinylated indicator, erythrocytes, or avidin, the following test is performed. Each candidate antibody-forming cell is picked up using a fine glass pipette and micromanipulator and is transferred to a second indicator system, again containing complement and enhancing serum, but in which antigen-coated sheep erythrocytes have been substituted with biotinylated sheep erythrocytes coated only with avidin. This indicator system is prepared as described above; if a plaque forms, the candidate cell is discarded as the antibody produced cannot be specific for the desired protein. If a plaque does not form in 40 minutes the cell is transferred to a warmed droplet containing the original indicator system, i.e., antigen-coated erythrocytes, complement and enhancing serum. Plaque formation

in 40 minutes is taken as confirmatory evidence that the candidate cell is indeed producing an antibody specific for the desired antigen.

Within an alternative embodiment, the indicator system contains equal numbers of antigen-coated erythrocytes and erythrocytes lacking the antigen (biotinylated erythrocytes coated only with avidin). Plaques formed by cells forming antibodies to the desired antigen will be "cloudy," as the non-antigen coupled erythrocytes will not be lysed. In contrast, any "background" plaques caused by cells fortuitously producing antibodies against non-antigen coupled erythrocytes will be clear as both coupled and uncoupled erythrocytes will be lysed.

Within another aspect of the present invention the indicator system may be used to identify antibody-forming cells, such as B lymphocytes, which produce antibodies of a desired specificity, but which express antibody on their surface rather than by secreting it.

For example, a "rosetting" procedure may be utilized to identify antibody-forming cells which produce antibodies that bind to a complexing factor, thus forming a rosette. The complexing factor may be sheep erythrocytes or other particles, such as polystyrene beads which have been coupled to an antigen. The erythrocytes or other particles may be coupled by any of the methods well known in the art, including the biotin/avidin coupling system discussed above. Alternatively, the complexing factor may be a microorganism such as a virus, parasite, yeast, or bacteria. To detect antibody-forming cells that bind to the desired particles, a population containing candidate antibody-forming cells, e.g., nucleated spleen cells from an immunized mouse, is mixed in medium with the particles. The mixture can be centrifuged at 200 g for 10 minutes and resuspended by gentle pipetting. Antibody-forming cells producing the desired antibody may be identified by the attached particles using inverted microscopy and picked up using a fine pipette and micromanipulators. Generally, it is preferable to first enrich the population of cells to which the particles have bound. For example, the complexes or rosettes which form due to the antigen-antibody interactions may also be enriched by various physical methods, including methods which are dependent on density, magnetic, fluorescent, or other properties of the rosettes. More specifically, rosettes formed by antibody-forming cells and coated erythrocytes may be enriched using a Ficoll-Hypaque gradient. (See C. R. Parish and J. A. Hayward, Proc. Roy. Soc. 13:117, 1974.) If the antigen is coupled to magnetic particles, cells binding the antigen-coated particles may be enriched by placing a magnet under part of a plastic flask or disk containing the cell

suspension and decanting cells that are not attracted to the magnet by bound particles. The individual antibody-forming cells in the rosettes may be identified under a microscope and isolated by micromanipulation.

Nonspecific antibody-forming cells may also be separated using the  
5 above techniques prior to the formation of rosettes with antigen-coated erythrocytes. Briefly, within one embodiment, a cell population containing B lymphocytes is mixed in medium with a suspension of control non-antigen-coated erythrocytes, e.g., coated with biotin and exposed to avidin and washed (without  
10 treatment with the biotin-coated antigen) at a final concentration of 5% by volume. The mixture is gently agitated at room temperature for 60 minutes. Rosettes consisting of B lymphocytes with adherent erythrocytes are separated from the remainder of the nucleated cells, for example by centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). This procedure depletes the population of B lymphocytes reactive with erythrocytes, biotin, or avidin. The  
15 procedure may then be repeated with erythrocytes coated with the antigen to enrich B lymphocytes reactive with that antigen. Individual antibody-forming cells that have bound the complexing factor (antigen-coated) erythrocytes may then be identified and isolated using an inverted microscope.

Other indicator systems may be employed to detect antibodies with  
20 a desired catalytic function. Within this aspect of the present invention, the indicator system comprises a substrate, and the antibody exhibiting a desired function is identified as that which modifies the substrate in a detectable manner. Within one embodiment, antibody-forming cells are obtained from animals immunized with chemical analogues of transition-states which occur during  
25 catalysis by enzymes. These chemical analogues are designed to mimic in their shape and charge distribution intermediate structures formed in the course of an enzymatic reaction (see R.A. Lerner and S.J. Benkovic, *Bioessays* 4:107-12, 1988). Lerner and colleagues (see B. L. Iverson and R. A. Lerner, *Science* 243:1184-8, 1989) have also described another approach wherein antibodies are raised against  
30 a complex including a substrate (a peptide) and cofactor (metal complex) in order to produce antibodies which catalyze specific hydrolysis of the glycine-phenylalanine peptide bond along with a metal complex cofactor.

Within another embodiment, antibody-forming cells that produce antibodies that activate or inactivate a protein such as an enzyme may be  
35 identified by including that protein in the indicator system. In one embodiment, a population of antibody-forming cells from an animal, e.g., a mouse immunized with an analogue or analogues of a transition-state occurring during catalysis of a

peptide bond, is mixed with an indicator system containing mouse erythrocytes and the precursor of the toxin, aerolysin, of *Aeromonas hydrophila* (J. T. Buckley et al., Can. J. Biochem. 59(6):430-5, 1981). Proteolytic cleavage of this precursor at a number of peptide bonds susceptible to a variety of known proteases activates the toxin (S. P. Howard and J. T. Buckley, J. Bacteriol. 163(1):336-40, 1985). Because the active toxin will lyse erythrocytes, antibody-forming cells secreting an antibody with proteolytic activity will be surrounded by a hemolytic plaque.

Within yet another embodiment, an antibody capable of activating plasminogen is identified using an indicator system in which antibody-forming cells, a gelling agent such as agar and nutrient medium are added to a mixture of plasma and a source of casein such as skim milk powder. In this indicator system, a cell that produces an antibody that activates plasminogen by cleaving it and generating plasmin is located by its central position in a plaque where the opacity of the skim milk is cleared by the local proteolysis of the casein by plasmin.

Within other embodiments, the indicator system may contain a chromogenic, fluorogenic, colored, or fluorescent substrate designed such that the function of the desired antibody, e.g., cleavage of the substrate, may be identified by the local generation or loss of color or fluorescence. For example, the action of the desired antibody could result in the local generation of a fluorescent or colored product or the local loss of a fluorescent or colored substance in the vicinity of the cell producing the antibody responsible for the cleavage. Within one embodiment, peptide substrates coupled with arido-4-methyl coumarin release a fluorescent compound when cleaved resulting in a ring of fluorescence surrounding the antibody-forming cell. Specific peptides can be used for different enzymes, e.g., N-acetyl-ala-pro-ala-7-amido-4-methyl coumarin is a specific fluorogenic substrate for elastase (see Zimmerman et al., Anal. Biochem. 78:47, 1977).

Antibodies which are labeled with fluorescent markers such as fluorescein or a phycobilliprotein may also be used as a tool to identify the products of the action of catalytic antibodies in the vicinity of an antibody-forming cell. Furthermore, a developing monoclonal antibody that recognizes and forms complexes with the products of the breakdown of fibrin (see M.J. Elms et al., Thromb. Res. 30:521, 1983) will form a precipitate around that antibody-forming cell and may thus be used to identify an antibody-forming cell secreting an antibody that cleaves fibrin.

Within yet another embodiment, a layer of gelatin may be utilized to detect an antibody with collagenase activity. Within this embodiment, gelatin

may be visualized by coupling it with a colored or fluorescent substance, e.g., by derivatizing it with dinitrophenol using dinitrophenylisothiocyanate (Eastman Kodak Limited, Rochester, N.Y.), to yield a yellow product, or with fluorescein using fluorescein isothiocyanate (Molecular Probes, Plano, Tex.) to yield a fluorescent product. This derivatized gelatin is overlaid with a thin layer of agar containing antibody-forming cells. After incubation at 37°C for one to four hours, the layer is washed with cold nutrient medium. A desired proteolytic antibody secreted by the antibody-forming cell will digest the gelatin into small fragments that may be washed away. Thus, areas lacking the colored or fluorescent gelatin are evidence of an antibody-forming cell which produces an antibody exhibiting the desired function.

In another embodiment, to detect an antibody capable of cleaving a peptide, that peptide is synthesized with an additional cysteine residue at the C-terminus and a biotin group at the N-terminus. The peptide is coupled by disulphide bonding to a convenient matrix with free thiol groups such as Thiol Sepharose beads, (Pharmacia, Uppsala, Sweden). Indicator groups such as fluorescein-labeled avidin may be used to bind to the biotin and thus label the peptide on the beads. The labelled-peptide beads are then incorporated with a cell population containing antibody-forming cells in a layer of nutrient medium gelled with 0.5% agar in a petri dish or on a glass slide. The slide is incubated at 37°C to allow an antibody with a proteolytic function to cleave the peptide, and thus to release the N-terminal fragment and its associated label. The agar is washed five times with 10 mls of cold 4°C nutrient medium, and visualized with fluorescent microscopy. Plaques or areas that fail to fluoresce because of the cleavage of the fluorescein labeled peptides in the vicinity of an antibody-forming cell are located, and the central antibody-forming cell producing the desired antibody is identified.

As discussed above, the antibody-forming cell producing the desired antibody is generally located by its appearance and central position in the indicator plaque. An inverted phase-contrast microscope is a convenient instrument for this purpose. The antibody-forming cell may then be removed and placed in nutrient medium or grown in culture. In most cases, the antibody-forming cell can easily be removed with a fine glass pipette and a micromanipulator, although with practice, a skilled technician may not require the micromanipulator. If more than one cell is present in the indicator plaque, the cells may be placed in a fresh indicator system and incubated for a further period in order to determine which cell produced the desired response.



Once an antibody-forming cell has been identified and isolated, the desired functional properties of an antibody may then be transferred from one antibody to another antibody or protein. This may be accomplished by transferring the variable region, or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell. Briefly, the special property of antibodies that allows the production by animals of a large variety of different antibody molecules, each with unique binding properties, is conferred by the presence in the antibody protein of two regions. One region, termed the "constant region," has the same structure in all antibodies from a particular isotype of the same species. The second region, termed the "variable region" (V-region), varies in its amino acid sequence from antibody to antibody, and it is this variability in structure that confers upon each particular antibody the special functional properties characteristic of that antibody. Antibody molecules are composed of two chains of amino acids (or polypeptides), each with a variable and constant region linked together by a disulphide bond. The larger chain is termed the "heavy chain" and the smaller the "light chain." The region of an antibody molecule that performs the desired function in the indicator systems described above (the "binding site") is made up of parts of both heavy and light chains. Thus, as discussed below, the sequences encoding the variable regions of the antibody produced by the antibody-forming cell may be transferred into another antibody or protein, thus conferring upon that antibody or protein the same function as that of the antibody produced by the isolated antibody-forming cell.

The variable regions of the light chain and the heavy chain each have three subregions where the variation in amino acid sequence between different variable regions is concentrated; these are termed "hypervariable regions." The amino acids that comprise these six hypervariable regions make up the binding site. The shape, charge, and other characteristics of the binding site determine the binding characteristics of the antibody and are termed the "complementarity determining regions" or CDRs. The key information that specifies the binding properties of an antibody is thus the amino acid sequence of the six CDRs, although in some cases the three CDRs of the heavy chain may confer a measurable and useful amount of specific binding for a particular protein. The amino acid sequence of the six CDRs is determined by the nucleotide sequence of corresponding parts of the gene encoding the variable regions of the heavy or light chains. Thus, the portion of the antibody produced by the antibody-forming cell that confers the desired function may be described largely by either

the amino acid sequence of the respective six CDRs or by the nucleotide sequence of the DNA that encodes and specifies these CDRs.

As will be evident to one of ordinary skill in the art, the regions of cDNA which encode the CDR may be identified by alignment of the predicated amino acid sequence of the V region in question with other V regions from the same species or V regions from other species. Methods of sequence comparison have been described by Kabat et al. (see "Sequences of Proteins of Immunological Interest," U.S. Department of Health and Human Services, 1987). Thus, although the binding characteristics of a given antibody may be transferred to another protein by incorporating into that protein the entire variable regions of the heavy and light immunoglobulin chains, substitution of only a portion of the variable region, such as the six CDRs, may successfully transfer or graft binding characteristics of antibody produced by the antibody-forming cell to another antibody or protein (see L. Reichmann et al., *Nature* 332:325-27, 1988). Furthermore, the successful transfer of the desired function to another protein or antibody may be confirmed by expressing that protein in a cell and testing that cell in the indicator system used to isolate the antibody forming cell.

The variable regions or a portion thereof, such as the complementarity determining regions from the isolated antibody may be incorporated into a protein such as an antibody utilizing at least two alternative methodologies:

(I) The amino acid sequence of the variable regions or portions of the variable regions such as the CDR from the antibody produced by the isolated antibody-forming cell may be determined, and used to construct another antibody or other protein with a function similar to that of the isolated antibody. Once this amino acid sequence information has been acquired, it may be used to synthetically construct proteins with a desired function, or the DNA sequence encoding the desired amino acid sequence may be incorporated in the form of synthetic oligonucleotides into a vector capable of expressing a protein with the desired function from a host cell. It will be understood by those skilled in the art that the nucleic acid sequence can be used to predict the amino acid sequence, and vice versa.

(II) Within a second methodology, RNA from the isolated antibody-forming cell is used to generate cDNA, and cDNA encoding the desired functional region of the antibody is amplified and directly incorporated into an expression vector. Alternatively, the RNA may be amplified, used to construct cDNA, and the cDNA then incorporated into an expression vector. Within either



of these alternatives, it is not necessary to determine the nucleic or amino acid sequence of the CDR, although in either case the RNA or DNA can be sequenced to determine the nucleotide sequences of the variable regions and, in particular, that of the respective CDRs. This information can be used to generate oligonucleotides that may be incorporated into a suitable vector. Alternatively, the information may be used to deduce amino acid sequences of the variable region and, in particular, the CDR and this information may be utilized to synthesize an antibody-like protein with the desired functional characteristics by chemical means.

#### I. AMINO ACID SEQUENCE DETERMINATION OF THE REGION CONFERRING THE DESIRED FUNCTIONAL PROPERTIES OF THE ANTIBODY PRODUCED BY THE ANTIBODY-FORMING CELL

Generally, within this method, the amino acid sequences of the variable regions or, more particularly, of the CDRs of the antibody produced by the isolated antibody-forming cell are determined, and incorporated into a selected protein. In general the binding properties of the antibody can be most confidently reproduced by reproducing the amino acid sequence of the entire heavy and variable regions of the light chains of the antibody molecule. However, in many cases, a useful protein with the binding properties of the antibody can be recreated by substitution of the amino acids of the CDRs of those variable regions for the CDRs of convenient heavy- and light-antibody chains. Reichman et al., in "Reshaping Human Antibodies for Therapy" (Nature 322:323-327), have described factors to be considered in choosing heavy- and light-chain polypeptides which may receive substituted CDRs in order to create another antibody with the same desired function as the antibody produced by the antibody-forming cell. Moreover, as described by Winter and colleagues (see E. S. Ward et al., Nature 341:544 1989; see also R. Orlandi et al., Proc. Natl. Acad. Sci. U.S.A. 86:3833 1989), in some cases significant capacity to bind antigen may reside in the CDRs of the heavy chain alone and dimers of heavy chains may bind usefully to antigens.

The amino acid sequence of the CDR may be determined by ...ee general methods: (1) directly from amino acid sequence of the antibody produced by the antibody-forming cell; (2) from the RNA of the antibody-forming cell; or (3) from the DNA of the antibody-forming cell.

**A. DETERMINATION OF THE VARIABLE REGION OR PORTION THEREOF FROM THE PARTIAL AMINO ACID SEQUENCE OF THE ANTIBODY PRODUCED BY THE ANTIBODY-FORMING CELL**

The amino acid sequence of the variable region or a portion thereof which confers the desired function may be determined directly by at least two different methods. First, the antibody-forming cell may be grown to a sufficient density such that sequenceable quantities of antibodies are produced. In brief, cells are incubated for 3 days in nutrient medium such as Iscoves Modified Dulbecco's medium containing bovine serum albumin. Supernatants are collected and the bulk of the bovine serum albumin is removed using bovine serum albumin-specific antibodies coupled to a solid matrix. The supernatant is then subjected to SDS-PAGE under reducing conditions. Proteins are transferred by electroblotting to a membrane such as Sequelon (Milligen/Bioscience, Boston, Mass.), and bands corresponding to the position of heavy- and light-immunoglobulin chains are sequenced, as described by Aebersold et al. ("Covalent Immobilization of Proteins for High-Sensitivity Sequence Analysis: Electroblotting Onto Chemically Activated Glass From Sodium Dodecyl Sulfate-Polyacrylamide Gels," Biochem. 27:6860, 1988). Alternatively, if the antibody-forming cell grows slowly or produces only small amounts of antibody, the cell may be immortalized with any of the techniques well known in the art, such as the EB virus, electrofusion, fusion with a myeloma, in addition to stimulation with factors such as IL-1, IL-2, IL-4, IL-5, and IL-6. Furthermore, in contrast to techniques relying on the production of stable antibody producing cells - e.g., the hybridoma technology or EBV-transformation technology for producing monoclonal antibodies, the present invention does not require stability beyond the period necessary to generate sufficient antibody for sequencing.

**B. DETERMINATION OF THE VARIABLE REGION OR PORTION THEREOF FROM THE RNA ENCODING PRODUCED BY THE ANTIBODY-FORMING CELL**

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Once an antibody-forming cell of a desired function has been identified, RNA is recovered from the cell by established methods, such as the method of Rappolee et al. (J. Cell Biochem. 39:1-11, 1989), or a scaled-down version of the method of Razin et al. (J. Immunol. 146:981-987, 1991). The amino acid sequence may then be determined from the RNA which is produced by the antibody-forming cell by at least two different methods.

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Within the first method, once RNA has been recovered, cDNA is constructed. Many methods for constructing cDNA from RNA are well known in the art, such as those described by Razin et al. (*J. Immunol.* 146:981-987, 1991).

Preferably, the cDNA is then amplified using techniques well known in the art, such as Polymerase Chain Reaction (PCR). See Mullis, U.S. Patent No. 4,683,195; Mullis et al., U.S. Patent No. 4,683,195; and Mullis et al., U.S. Patent No. 4,800,159. See also H. A. Erlich (ed.), *PCR Technology*, Stockton Press, New York, N.Y., 1989; M. A. Innis et al. (eds.), *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, Calif., 1989; H. A. Erlich (ed.), *Polymerase Chain Reaction: Current Communication in Molecular Biology*, Cold Springs Harbor Press, Cold Spring Harbor, N.Y., 1989. Briefly, cDNA segments encoding the portion of the antibody that confers the desired function are exponentially amplified by performing sequential reactions with a DNA polymerase. The reaction is primed by a 5' and a 3' DNA primer, the 3' antisense primer corresponding to a DNA sequence in the constant (or joining) region of the immunoglobulin chain and the 5' primer (or panel of related primers) corresponding to a DNA sequence in the variable region of the immunoglobulin chain. This combination of oligonucleotide primers has been used in the PCR amplification of murine immunoglobulin cDNAs of unknown sequence (see Sastry et al., *Proc. Natl. Acad. Sci.* 86:5728-5732, 1989; and Orlandi et al., *Proc. Natl. Acad. Sci.* 86:3833-3837, 1989). When immunoglobulin cDNAs are amplified from species in which it is difficult to design adequate 5' primers, i.e., where few immunoglobulin variable region sequences are known, an "anchored polymerase chain reaction" may be performed (see Loh et al., *Science* 243:217-220, 1989). In this procedure, the first strand cDNA is primed with a 3' DNA primer as above, and a poly(dG tail) is then added to the 3' end of the strand with terminal deoxynucleotidyl transferase. The product is then amplified by PCR using the specific 3' DNA primer and another oligonucleotide consisting of a poly(dC) tail attached to a sequence with convenient restriction sites.

The amino acid sequence encoding the portion of the antibody that confers the desired function may also be determined by a second method. The recovered RNA is first amplified with T7 or Q- $\beta$  replicase (see Lizardi et al., *Bio/Technology* 6:1197-1202, 1988). Briefly, a primer bearing a T7 promoter sequence, the left half of the MDV-1 sequence (the naturally occurring template for Q- $\beta$  replicase) and a homologous sequence of the specific RNA being amplified is used to produce a cDNA copy of the specific RNA using an appropriate DNA polymerase. This extended first DNA is hybridized to a second

DNA containing the right half of the MDV-1 sequence and a sequence homologous to the extended sequence of the first DNA. The second sequence is extended with a DNA polymerase. Incubation of these DNA complexes with T7 RNA polymerase results in synthesis of recombinant RNAs that can amplify exponentially by incubation with Q- $\beta$  replicase. Once the amplified RNA has been recovered, cDNA may be constructed using the above-described methods.

Once the cDNA has been obtained using either of the above two methods, the nucleotide sequence may then be determined by established conventional methods. The amino acid sequence of the CDRs will then be evident from translation of the nucleotide sequence.

#### C. DETERMINATION OF THE VARIABLE REGION OR A PORTION THEREOF FROM THE DNA ENCODING THE ANTIBODY PRODUCED BY THE ANTIBODY-FORMING CELL

The amino acid sequence encoding the portion of the antibody that confers the desired function may also be determined from the DNA of the antibody-forming cell. Briefly, DNA is recovered from the cell using standard procedures. Clonal expansion with growth factors or fusion or immortalization of the cell as discussed above will enhance the yield of DNA. The specific DNA sequences encoding the rearranged V-regions including the CDRs are amplified by PCR and resulting DNA sequenced by standard procedures. The amino acid sequence of the variable region or portion thereof may be determined by translation of the nucleotide sequence of the DNA. Because this strategy may occasionally amplify a nonproductively rearranged V-region, it may be necessary to test the expression combination of the different variable heavy and light chains in a suitable cell and test for the production of the desired antibody in the indicator system.

Once the amino acid sequence of the variable region or portion thereof has been identified, the predicted DNA sequence corresponding to that of the amino acid sequence may be incorporated into a vector capable of directing the expression and secretion of the protein with a desired function.

The procedure described by Reichman et al. in "Reshaping Human Antibodies for Therapy," Nature 322:323-327, may be followed to substitute the portion of the antibody that confers the desired function for those of selected heavy- and light-immunoglobulin chain genes. Briefly, large mutagenic oligonucleotides corresponding to the complementarity determining regions of the

heavy and light chains of the antibody in question are transferred to plasmids that contain genomic clones corresponding to a rearranged heavy-chain gene or rearranged light-chain gene, respectively.

The resultant heavy- and light-chain genes, now bearing the complementarity determining regions, may be expressed by the vectors in host cells by methods well known to those skilled in the art. Many suitable host cells may be utilized, including among others, bacterial, fungal, mammalian, and insect cells. The cell type (such as COS cells or CHO cells) may be selected according to the nature of the expression vector employed such that expression of the immunoglobulin is optimized. The cells are then transfected using standard methods which are well known to one of ordinary skill in the art. These methods include electroporation or transfection using calcium phosphate, DEAE dextran, or protoplast fusion methodologies (see Sambrook et al. *supra*.) Subsequently, proteins which are expressed by the host cell may be readily purified utilizing methods well known in the art (see generally, Sambrook et al. *supra*).

Alternatively, as discussed above, once the amino acid sequence of the portion of the antibody that confers the desired function has been determined, a protein may be synthesized directly, such that the synthetically constructed protein contains the amino acid sequence of the conferring the desired function. Methodologies for the efficient chemical synthesis of large proteins have been optimized by Clark-Lewis et al. (see Clark-Lewis et al., *Science* 231:134-139, 1986). A particularly preferred method is to synthesize and refold separately the variable regions of the heavy and light chains using the methods outlined by Clark-Lewis and colleagues (see Clark-Lewis, *supra*). Subsequently, the proteins are allowed to associate to form heavy chain - light chain dimers under conditions as described by E. A. Kabat in Structural concepts in Immunology and Immunochemistry, Holt, Rinehart & Winston, Inc., New York, N.Y., 1968, pp. 157-81.

Within one embodiment of the invention, the amino acid sequence may be optimized to enhance the desired function of the protein. Reichman et al. ("Reshaping Human Antibodies for Therapy," *Nature* 322:323-327) have outlined some of the factors to be considered in altering the amino acid sequence of the variable region. Additionally, the indicator systems described herein are powerful tools for screening proteins with variations in their amino acid sequence to identify variants with enhanced functional activities. For example, cells transfected with vectors with mutations that lead to changes in the amino acid sequence of the CDRs or other parts of the V-region and thus secreting proteins

with potentially enhanced functional activity may produce larger plaques in the appropriate indicator system.

The desired function of the antibody produced by the antibody-forming cell may also be transferred into proteins other than complete immunoglobulin molecules. These proteins include active fragments of immunoglobulin molecules and analogs of immunoglobulins which are also able to provide a functional framework for the CDRs. These proteins may be expressed either in mammalian cells (see L. Reichman and J. Foote, J. Molec. Biol. 203:825-828, 1988) or in bacteria (see A. A. Sperra and A. Pluckthin, Science 240:1038-1041, 1988). Single chain molecules comprising the variable regions of heavy and light chains bridged by a polypeptide and produced as a single polypeptide (see J. S. Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883, 1988) are another type of protein into which desired binding characteristics can be introduced by the present invention. Likewise, the desired binding characteristics can be transferred by this invention to fusion proteins in which the V-regions of heavy and light chains are fused to one another and to other functional domains or proteins such as toxins (see Vijay K. Chaudhary et al., Nature 339:394, 1989).

Vectors may also be produced which direct the expression of a protein with a desired function within a host cell, but in which the nucleotides encoding the signal sequences that direct secretion of the translated protein have been deleted. Such a vector will only direct expression of the protein within the cytoplasm of the host cell, and not secretion of the protein (see S. Biocca et al., EMBO J. 9(1):101-108, 1990). Suitable vectors directing the production of intracellular antibodies or antibody derived proteins into which the binding or other functional characteristics of an antibody have been introduced are useful, for example, in inhibiting the action of a particular protein in a cell and its progeny. Proteins that may be targeted include viral proteins essential for infection by the virus, so that by this means cells of animal or plant origin can be made resistant to infection by a virus, for example, HIV-1. Uses include the introduction of such vectors into the germline to produce disease-resistant transgenic animals or plants or into hemopoietic stem cells to produce an immune system in which T lymphocytes and other cells of hemopoietic origin are resistant to infection with a virus such as HIV.

## II.

Within a second methodology, as described in more detail below in the Examples, the variable region or a portion thereof (for example, the heavy- and light-chain variable regions) are subcloned into expression vectors designed to restore an appropriate leader sequence and an immunoglobulin-constant region of the chosen light- or heavy-chain class. Alternatively, the amplified cDNA corresponding to complete heavy and light chains is cloned and subcloned into an expression vector restoring a leader sequence. Such an expression vector would be based on an established vector into which a functional leader sequence had been introduced. Ligation of the light or heavy chains, their variable regions or portions thereof into this expression vector would reconstitute a functional signal peptide.

As will be understood by one of ordinary skill in the art, components necessary to practice the present invention may be provided in kit form. For example, within one embodiment a kit suitable for carrying out the above-described invention includes: cell lysis buffer (see Example 3), enzymes for reverse transcription and PCR, as well as the appropriate buffers (see Example 3), a set of all primers (see Example 3, SEQ ID.Nos: 1-12), and a vector such as p587L. Although these components are provided by way of a representative example, as one of ordinary skill in the art will appreciate various components of the present invention may be interchanged with those known to one of ordinary skill in the art.

The following examples are offered by way of illustration, and not by way of limitation.

**EXAMPLES**

30

**EXAMPLE 1****DEVELOPMENT OF AN INDICATOR SYSTEM TO DETECT AN ANTIBODY-FORMING CELL WHICH  
NEUTRALIZES HUMAN IL-2**

To identify an antibody that is able to neutralize the growth factor human interleukin-2, a cell-line such as CTLL-2 (ATCC No. TIB 214) that requires human IL-2 to live in culture systems, is washed three times and incubated for 4 hours at 37°C in medium, washed again and suspended at  $10^8$  per

- ml in warm (37°C) medium containing RPMI-1640, 10% fetal bovine serum to which has been added a 1/10 volume of a sterile warm (40°C) solution of 3% agar (DIFCO Laboratories, Mich.) in water, together with about 0.5 unit of human IL-2 (Amgen, Sevenoaks, Calif.). This medium promotes 50% of maximal proliferation
- 5 of this concentration of indicator cells and maintains completely their survival for 24 hours. A source of factors that can enhance the *in vitro* survival of antibody-forming cells can be included; one such useful preparation for mouse antibody-forming cells is 10% by volume of a tenfold concentrate of medium conditioned by the murine cell-line 3T3 (ATCC No. CCL 92). Other useful additives are IL-1, IL-
- 10 2, GM-CSF, IL-4, IL-5, IL-6 and interferon- $\gamma$ , usually of the species from which the antibody-secreting cells were derived. Antibody-forming cells are prepared from the spleen of a mouse immunized intraperitoneally three times at monthly intervals with human interleukin-2, 100  $\mu$ g in 0.1  $\mu$ l of Freund's incomplete adjuvant (Difco Laboratories, Mich.), and on the first occasion, in 0-1 ml of
- 15 Freund's complete adjuvant (Difco Laboratories, Mich.). The presence of anti-IL-2 antibodies in the serum is confirmed by testing serum from the mouse for the capacity to inhibit IL-2 dependent proliferation of an IL-2 dependent cell-line such as CTLL-2, and to bind IL-2 in an ELISA assay. A mouse with the highest titres of IL-2 neutralizing activity is selected and is injected intravenously with 10
- 20  $\mu$ g of interleukin-2. Four to seven days later, the spleen is removed and gently dissociated into a cell-suspension. Erythrocytes are lysed by brief exposure to 0.75% hypotonic ammonium chloride in 0.017 M Tris buffer (pH 7.2), leaving only nucleated spleen cells ("Selected Methods in Cellular Immunology," B. Mishell and S. Shiigi (eds.), W. H. Freeman and Co., San Francisco, 1980.
- 25 The nucleated spleen cells are added to the indicator system at final concentrations ranging from  $10^4$ /ml to  $10^5$ /ml. One ml of the mixture is applied to a warm (37°C) glass slide or petri dish and after briefly storing at room temperature or in a cold room at 4°C to allow the agar to set, the slide is incubated overnight at 37°C in a humidified CO<sub>2</sub> incubator. The preparation is
- 30 examined for the presence of plaques of dead cells, using an inverted microscope and phase contrast optics. Antibody-forming cells that have secreted a neutralizing antibody into their immediate environment are identified as central viable cells in plaques of dead cells.



### EXAMPLE 2

#### DEVELOPMENT OF AN INDICATOR SYSTEM TO DETECT CELLS WHICH MIMIC INTERLEUKIN-2

To detect cells producing antibodies that mimic IL-2, cells that  
5 require IL-2 to survive such CTLL-2's, are incubated at a density of  $2 \times 10^6$  cells  
per square centimetre, together with a source of nucleated spleen cells taken from  
an animal, e.g., a rat, immunized against the receptor for IL-2. This can be  
accomplished by immunization by standard techniques with CTLL-2, or a purified  
10 preparation of the receptor. The indicator system mixture is prepared with 0.3%  
agar and CTLL-2 indicator cells and a source of antibody-secreting cells as  
described above, except that no viability-sustaining factor such as IL-2 is included,  
and is layered on a transparent surface and incubated as described above. Cells  
producing antibodies that mimic IL-2 and produce the desired effect, in this  
15 instance of maintaining viability of the indicator cells, may be identified within  
plaques of surviving cells.

### EXAMPLE 3

#### PRODUCTION OF AN ANTI-ERYTHROCYTE ANTIBODY

20 A Balb/C mouse is immunized by intraperitoneal injection of  $10^9$   
sheep erythrocytes suspended in 0.2 ml of normal saline. Five days later, the  
mouse is sacrificed and the spleen removed. A single-cell suspension is prepared  
by gently mincing the spleen with scissors and pressing it through a wire sieve.  
The spleen cells are washed once with tissue-culture medium (RPMI-1640 with  
25 10% fetal calf serum and 20 mM HEPES) and resuspended in 40 ml of this  
medium.

A mixture is prepared, consisting of spleen cells at  $10^5$ - $10^6$  per ml, a  
source of complement such as 15% (v/v) guinea pig serum preabsorbed twice with  
an equal volume of sheep erythrocytes, sheep erythrocytes at  $5 \times 10^8$ /ml, and an  
30 enhancing serum, e.g., 0.5% sheep anti-mouse immunoglobulin) in nutrient  
medium buffered with 20mM HEPES. One to ten microliter aliquots of this  
mixture are introduced under a thin layer of paraffin oil (warmed to  $37^\circ\text{C}$ ) on a  
warm ( $37^\circ\text{C}$ ) glass microscope slide which was incubated in a humidified incubator  
at  $37^\circ\text{C}$  in 10%  $\text{CO}_2$  and air for 30 minutes.

35 The slide is then placed on the stage of an inverted microscope.  
Areas where the sheep erythrocytes have been lysed by the action of antibody and  
complement ("plaques") are located under phase-contrast illumination. Plaques

which the antibody-forming cell has formed can then be confidently identified through the following characteristics:

- (a) Evidence of lysis rather than artifactual clearing of the erythrocytes in the form of lysed erythrocyte "ghosts."
- 5 (b) The presence in the plaque of a single, centrally located viable cell.

The ability of the identified cell to produce the desired antibody is confirmed by transferring it (using a fine glass pipette manipulated by a micromanipulator and connected to a syringe controlled by a micrometer) first  
10 into a droplet of medium on the slide to dilute out any carried-over antibody and a second time into a droplet containing sheep erythrocytes, guinea pig serum, and medium with enhancing serum as before (but no other cells) to confirm that this cell formed another plaque after incubation for 15 minutes.

This cell is then transferred (in a volume no greater than 20  $\mu$ l of  
15 culture medium) to an eppendorf tube. To this tube is added 20  $\mu$ l of Proteinase K (BDH Biochemicals) at 10mg/ml and 100 $\mu$ l of lysis buffer (10mM Tris HCl pH 7.4, 100mM NaCl, 1mM EDTA, 0.5% SDS). The solution is immediately vortexed vigorously then incubated at 37°C for 60 minutes.

After this incubation a further 20  $\mu$ l of 2.5M NaCl is added together  
20 with 50  $\mu$ g of oligo-dT cellulose, and the mixture incubated overnight on a rotating wheel. The oligo-dT cellulose (and the bound polyadenylated RNA) is then pelleted by centrifugation, washed once with a wash buffer (10mM Tris HCl pH 7.4, 500mM NaCl, 1mM EDTA, 0.5% SDS), the supernatant discarded then the pellet washed again with a buffer comprising 100mM NaCl and 10mM Tris  
25 HCl (pH 7.4).

The polyadenylated RNA is then eluted from the oligo-dT cellulose by resuspension of the pellet in 15  $\mu$ l of 10mM Tris HCl (pH 7.4). The oligo-dT cellulose is separated by centrifugation and the supernatant retained. The eluted RNA is then reverse-transcribed in a buffer consisting of 50mM Tris HCl pH 8.3,  
30 75mM KCl, 10mM DTT, 3mM MgCl<sub>2</sub>, 0.5mM each of dGTP dATP dTTP and dCTP, 200u M-MLV reverse transcriptase and 1mM of each specific antisense oligonucleotide primer listed below. This reaction is performed in a total volume of 25  $\mu$ l at 37°C for 60 minutes.

For the following PCR reactions the polyadenylated RNA is divided  
35 into five pools. The first pool contains the sense and antisense oligonucleotide primers for the Kappa light chain. The remaining four pools each contain the antisense oligonucleotide primer specific for the CH1 regions of the Gamma 1

heavy chain as well as two (of the eight) sense oligonucleotide primers which are specific for the variable region of the Gamma heavy chain.

The antisense (and sense) primers are designed to amplify the variable and constant regions of the immunoglobulin heavy chains of classes mu and gamma, and the variable and constant regions of the immunoglobulin light chains of class kappa. The antisense primers used correspond to the 3' ends of the coding regions of the heavy and light chain mRNAs. A HindIII site is introduced at the stop codon to facilitate cloning of the PCR fragment into a pUC 12 vector.

These antisense oligonucleotide primers would be:

1. A primer for a Kappa light-chain constant region (SEQ ID. NO:1):

5' TAG AAG CTT CTA ACA CTC ATT CCT GTT GAA GCT CTT GAC 3'

2. A primer specific for the Gamma CH1 regions (SEQ ID. NO:2):

5' TTT GG(AG) GGG AAG ATG AAG AC(GA) GAT G(GA)T (CA)C 3'

The entire 25  $\mu$ l from the reverse priming reaction is then transferred to a tube containing 25mM TAPS pH 9.3, 2mM MgCl<sub>2</sub>, 50mM KCl, 1mM dithiothreitol, 2.5u Taq DNA polymerase, 0.2mM of each dATP, dCTP, dGTP and dTTP, and 1mM of each specific sense oligonucleotide primer. The total volume for this reaction is 100  $\mu$ l.

The sense oligonucleotide primers recognize regions of the variable region DNAs encoding the N terminal residues of the mature light and heavy chains. A HindIII site is introduced at the 5' end of each oligonucleotide primer to facilitate cloning of the PCR fragment into a pUC 12 vector.

These oligonucleotide primers would be:

1. A primer recognizing all Kappa variable regions (SEQ ID. NO:4):

5' TAC AAG CTT CCA GA(TG) GTG ACA T(CT)(AT) (AT)GA TGA CCC AGT CTC CA 3' [Interchangeable nucleotides are indicated in parentheses]

2. A series of primers together able to recognize all heavy chain variable regions (listed from 5' to 3'):

SEQ ID. NO:5 TAC AAG CTT CCG AGG TCC AGC TGG TGG AGT CTG G  
 SEQ ID. NO:6 TAC AAG CTT CCG AGG TCC AGC TGG TGG AGT CAG G  
 SEQ ID. NO:7 TAC AAG CTT CCG AGG TCC AGC TTG TGG AGT CTG G  
 SEQ ID. NO:8 TAC AAG CTT CCG AGG TCC AGC TTG TGG AGT CAG G  
 5 SEQ ID. NO:9 TAC AAG CTT CCG AGG TCC AAC TGG TGG AGT CTG G  
 SEQ ID. NO:10 TAC AAG CTT CCG AGG TCC AAC TGG TGG AGT CAG G  
 SEQ ID. NO:11 TAC AAG CTT CCG AGG TCC AAC TTG TGG AGT CTG G  
 SEQ ID. NO:12 TAC AAG CTT CCG AGG TCC AAC TTG TGG AGT CAG G

10 The tubes are then placed in an automated thermal cycler, such as the Perkin-Elmer Cetus DNA Thermal Cycler (Norwalk CT, Part No. N801-0055) and cycled for 30 cycles with a standard profile using an annealing temperature of 50°C. The reaction mix containing the amplified Kappa light chain is then ethanol precipitated in the presence of 5 µg tRNA, resuspended in a total volume of 100 µl and digested to completion with HindIII.

15 Five microliters of the reaction mixtures containing the amplified heavy chain DNAs are then subjected to a further two cycles at 60°C in the automated thermal cycler, then for 19 additional cycles at 70°C. For these additional PCR cycles the eight sense oligonucleotide primers described above for 20 the heavy chain variable region are again utilized in four groups of two, together with the following nested oligonucleotide primer which is specific for another portion of the CH1 region of all gamma chain constant regions (SEQ ID. NO:3):

25 5' TAC AAG CTT CTA ACA ATC (TC)CT (GA)GG CAC (AG)A(TG)  
 T(TC)T (CT)TT G(TA)(CT) CA(CA)C 3'

The reaction mixes containing the amplified gamma light chain are then ethanol precipitated in the presence of 5 µg tRNA, resuspended in a total volume of 100 µl and digested to completion with HindIII.

30 The digested light chain and heavy chain DNAs are then separated by 1% agarose gel electrophoresis and the amplified light- and heavy-chain DNAs isolated from the gel. The light-chain DNA would be expected to be in the order of 600-700 bp and the heavy-chain DNA in the order of 600-750 bp.

Each of the light and heavy chains is cloned into the HindIII site of 35 pUC12 by standard methods. (See Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989). Each end of both chains is sequenced by double stranded sequencing using the standard dideoxy chain termination method. The sequencing has four purposes: first, to

confirm that the bands are indeed immunoglobulin heavy and light chains; second, that the 5' primer has introduced the HindIII in the required frame (see below); third, that the 3' primer has introduced an in-frame stop codon; fourth, to locate convenient restriction sites with which to determine orientation of the insert when subcloned into the expression vector.

The light- and heavy-chain DNA sequences are then transferred from the pUC12 vector to the HindIII site of the expression vector p587L (see Gunning et al., Proc. Natl. Acad. Sci. 84:4831-4835, 1987) using standard methods. This vector is based on the p587 expression vector in which the leader sequence of the murine IL-3 gene has been inserted. Ligation of the light or heavy chain into this p587L vector, in the correct orientation, will reconstitute a functional signal peptide. Orientation is determined by digestion with the restriction endonuclease found to be convenient on DNA sequencing, followed by separation of the digested fragments by agarose gel electrophoresis.

The separate light- and heavy-chain expression constructs are expanded in *E. coli* and purified by standard methods. These two expression constructs are then mixed in equimolar amounts and transfected into COS-7 cells for expression. This transfection is performed by a standard calcium phosphate precipitation method. Twelve hours after transfection, the culture medium is changed and the cells cultured for a period of 3 days.

Conditioned medium from these cultures is collected and the protein is purified using an immuno-affinity column coupled with antibodies specific for the protein. Such antibodies could include antibodies specific for the variable region, framework region, or part of the constant region of immunoglobulin derived from the same species as the DNA sequences in the vector into which oligonucleotides corresponding to the CDRs of the prototypic had been inserted. Affinity chromatography using a column coupled with the substance to which the antibody binds may also be utilized to purify the antibody.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Schrader, John W.
- (ii) TITLE OF INVENTION: METHODS FOR THE PRODUCTION OF PROTEINS  
WITH A DESIRED FUNCTION
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Seed and Berry
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  - (C) CITY: Seattle
  - (D) STATE: Washington
  - (E) COUNTRY: U.S.
  - (F) ZIP: 98104-7092
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
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  - (C) REFERENCE/DOCKET NUMBER: 140053.401PC
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  - (C) TELEX: 3723836

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAGAAGCTTC TAACACTCAT TCCTGTTGAA GCTCTTGAC

39

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTGGRGGGA AGATGAAGAC RGATGRTMC

29

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TACAAGCTTC TAACAATCYC TRGGCACRAK TYTYTTGWYC AMC

43

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TACAAGCTTC CAGAKGTGAC ATYWWGATGA CCCAGTCTCC A

41

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TACAAGCTTC CGAGGTCCAG CTGGTGGAGT CTGG

34

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TACAAGCTTC CGAGGTCCAG CTGGTGGAGT CAGG

34

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA



(iii) HYPOTHETICAL: NO

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TACAAGCTTC CGAGGTCCAG CTTGTGGAGT CTGG

34

(2) INFORMATION FOR SEQ ID NO:8:

- (1) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(111) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TACAAGCTTC CGAGGTCCAG CTTGTGGAGT CAGG

34

(2) INFORMATION FOR SEQ ID NO:9:

- (1) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(111) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TACAAGCTTC CGAGGTCCAA CTGGTGGAGT CTGG

34

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TACAAGCTTC CGAGGTCCAA CTGGTGGAGT CAGG

34

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TACAAGCTTC CGAGGTCCAA CTGGTGGAGT CAGG

34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACAAGCTTC CGAGGTCCAA CTTGTGGAGT CTGG

34

Claims

1. A method for producing a protein with a desired function, comprising:

(a) providing a population of antibody-forming cells suspected of containing at least one cell capable of producing an antibody exhibiting the desired function;

(b) suspending the population of antibody-forming cells in a medium, the medium having an indicator system incorporated therein, said indicator system being capable of indicating the presence and location of a cell which forms antibodies exhibiting the desired function;

(c) identifying a cell forming an antibody exhibiting the desired function;

(d) isolating the identified antibody-forming cell from the medium;

(e) determining the amino acid sequence of the variable region, a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell; and

(f) synthesizing a protein with a desired function, said protein containing the amino acid sequence of said the variable region or portion thereof which confers the desired function.

2. A method for producing a protein with a desired function, comprising:

(a) providing a population of antibody-forming cells suspected of containing at least one cell capable of producing an antibody exhibiting the desired function;

(b) suspending the population of antibody-forming cells in a medium, the medium having an indicator system incorporated therein, said indicator system being capable of indicating the presence and location of a cell which forms antibodies exhibiting the desired function;

(c) identifying a cell forming an antibody exhibiting the desired function;

(d) isolating the identified antibody-forming cell from the medium;

(e) determining the amino acid sequence of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell;

(f) incorporating the DNA sequence corresponding to the amino acid sequence of said variable region or portion thereof conferring the desired function into a vector, said vector capable of directing the expression and secretion of said protein with a desired function;

- (g) transfecting said vector into a host cell;
- (h) growing said host cells in an appropriate medium; and
- (i) isolating the protein with the desired function.

3. The method of claims 1 or 2 including, subsequent to the step of determining the amino acid sequence, optimizing the amino acid sequence of said variable region or portion thereof conferring the desired function such that the desired function is enhanced.

4. The method of claims 1 or 2 including, subsequent to the step of isolating the antibody-forming cell:

- (a) recovering RNA from said isolated antibody-forming cell;
- (b) generating cDNA from said RNA; and
- (c) amplifying the cDNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell.

5. The method of claims 1 or 2 including, subsequent to the step of isolating the antibody-forming cell:

- (a) recovering RNA from said isolated antibody-forming cell;
- (b) amplifying the RNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell; and
- (c) generating cDNA from said RNA.

6. The method of claims 1 or 2 including, subsequent to the step of isolating the antibody-forming cell:

- (a) recovering DNA from said isolated antibody-forming cell; and
- (b) amplifying the DNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell.

7. A method for producing a protein with a desired function, comprising:

(a) providing a population of antibody-forming cells suspected of containing at least one cell capable of producing an antibody exhibiting the desired function;

(b) suspending the population of antibody-forming cells in a medium, the medium having an indicator system incorporated therein, said indicator system being capable of indicating the presence and location of a cell which forms antibodies exhibiting the desired function;

(c) identifying a cell forming an antibody exhibiting the desired function;

(d) isolating the identified antibody-forming cell from the medium;

(e) recovering RNA from said isolated antibody-forming cell;

(f) generating cDNA from said RNA;

(g) amplifying the cDNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell;

(h) incorporating the amplified cDNA into a vector capable of directing the expression and secretion of a protein with the desired function;

(i) transfecting said vector into a host cell;

(j) growing said host cells in an appropriate medium; and

(k) isolating the protein with the desired function from said host cell.

8. A method for producing a protein with a desired function, comprising:

(a) providing a population of antibody-forming cells suspected of containing at least one cell capable of producing an antibody exhibiting a desired function;

(b) suspending the population of antibody-forming cells in a medium, the medium having an indicator system incorporated therein, said indicator system being capable of indicating the presence and location of a cell which forms antibodies exhibiting the desired function;

(c) identifying a cell forming an antibody exhibiting the desired function;

(d) isolating the identified antibody-forming cell from the medium;

(e) recovering RNA from said isolated antibody-forming cell;

(f) amplifying the RNA of the variable region or a portion thereof which confers the desired function of the antibody produced by the isolated antibody-forming cell;

(g) generating cDNA from said RNA;

(h) incorporating the cDNA into a vector capable of directing the expression and secretion of a protein with the desired function;

(i) transfecting said vector into a host cell;

(j) growing said host cells in an appropriate medium; and

(k) isolating the protein with the desired function from said host cell.

9. The method of claims 1, 2, 7, or 8 wherein said medium is selected from the group consisting of a layer of gelified media and a layer of liquid media.

10. The method of claim 9 wherein the medium is a nutrient medium.

11. The method of claim 9 wherein the gelified media comprises about 0.3% to 0.6% agar or about 1.0% methyl cellulose.

12. The method of claims 1, 2, 7, or 8 wherein the antibody-forming cells are obtained from an animal which has been immunized with a selected antigen.

13. The method of claim 12 wherein the antigen is selected from the group consisting of erythropoietin, erythropoietin receptors, colony-stimulating factors, and viral antigens.

14. The method of claims 1, 2, 7, or 8 wherein the antibody-forming cells are obtained from an animal which generated said cells during the course of a selected disease.

15. The method of claims 1, 2, 7, or 8 wherein said animal is a warm-blooded animal selected from the group consisting of rabbits, humans, mice, rats, sheep, cows, and pigs.

16. The method of claims 1, 2, 7, or 8, including, subsequent to the step of isolating an identified antibody-forming cell, enriching the population of antibody-forming cells by exposure to bovine serum albumin or Percoll gradient.

17. The method of claims 1, 2, 7, or 8 wherein the indicator system comprises a layer of cells whose growth, viability, or function is affected by antibodies exhibiting a desired function produced by the isolated antibody-forming cell.

18. The method of claims 1, 2, 7, or 8 wherein the antibody which exhibits a desired function exhibits biological activity analogous to a protein selected from the group consisting of IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IFN- $\alpha$ , IFN- $\gamma$ , G-CSF, M-CSF, GM-CSF, TNF- $\alpha$ , TGF- $\beta$ , erythropoietin, EGF, PDGF, and the ligand of the kit protein.

19. The method of claims 1, 2, 7, or 8 wherein the indicator system comprises one or more pathogenic microorganisms, and a layer of cells susceptible to infection by said microorganisms, wherein said antibodies exhibiting a desired function are identified as those which effect the pathogenicity of the microorganism.

20. The method of claim 19 wherein the layer of cells is adhered to a glass slide or petri dish.

21. The method of claims 1, 2, 7, or 8 wherein the indicator system comprises a set of two cell types selected from the group consisting of cells of distinct HLA histocompatibility antigen types, blood group antigen types, and tumor cells and normal cells of the same lineage, wherein said antibodies exhibiting a desired function are identified as those which agglutinate or lyse one cell of the pair.

22. The method of claim 21 wherein one of said cell types is labeled.

23. The method of claims 1, 2, 7, or 8 wherein the indicator system comprises erythrocytes or other particles coated with antigen, and said antibody exhibiting a desired function is identified as that which binds to the antigen, thereby causing the particles to agglutinate.

24. The method of claims 1, 2, 7, or 8 wherein the indicator system comprises erythrocytes or other particles coated with antigen, and said antibody exhibiting a desired function is identified as that which binds to the antigen, lysing the cells or particles in the presence of complement.

25. The method of claim 24 wherein the erythrocytes or other particles are coated with antigen utilizing a biotin/avidin coupling system.

26. The method of claim 24 wherein the indicator system further comprises anti-immunoglobulin antibodies such that complement fixation is enhanced.

27. The method of claims 1, 2, 7, or 8 wherein the indicator system comprises a complexing factor, and said antibody-forming cell is identified as that which binds to the complexing factor, forming a rosette.

28. The method of claim 27 wherein the complexing factor is an erythrocyte coupled to an antigen.

29. The method of claim 27 wherein the complexing factor is a particle coupled to an antigen.

30. The method of claim 27 wherein the complexing factor is a microorganism.

31. The method of claims 1, 2, 7, or 8 wherein the indicator system comprises a substrate, and said antibody exhibiting a desired function is identified as that which modifies the substrate in a detectable manner.

32. The method of claim 31 wherein the substrate is a chromogenic substrate, and wherein said chromogenic substrate is modified to produce a detectable color change in the vicinity of the antibody-forming cell.

33. The method of claim 31 wherein the substrate is a fluorogenic substrate, wherein said fluorogenic substrate is modified to produce a detectable change in fluorescence in the vicinity of the antibody-forming cell.

34. The method of claim 31 wherein the substrate is a colored substrate, wherein said colored substrate is modified to produce a detectable loss of color in the vicinity of the antibody-forming cell.



35. The method of claim 31 wherein the substrate is a fluorescent substrate, and wherein said fluorescent substrate is modified to produce a detectable loss of fluorescence in the vicinity of the antibody-forming cell.

36. The method of claim 31 wherein the antibody exhibiting a desired function cleaves the substrate, the product of this reaction allowing the detection of said antibody upon the addition of a reagent selected from the group consisting of stains, and, labeled antibodies.

37. The method of claim 31 wherein the substrate is a precursor of the hemolytic toxin aerolysin and wherein said antibody exhibiting a desired function is identified as that which cleaves said precursor, thus activating the toxin.

38. The method of claims 1, 2, 7 or 8 wherein the indicator system is positioned contiguous to the medium.

39. A protein produced according to the method of claims 1, 2, 7 or 8.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/CA 91/00275

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K15/00; C12Q1/18; A61K39/395

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12Q

Documentation Searched other than Minimum Documentation  
to the extent that such Documents are included in the Fields Searched<sup>8</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	FR,A,2 485 739 (INSTITUT PASTEUR) 31 December 1981 see page 6, line 8 - line 17; claim 11	1-39
Y	JOURNAL OF IMMUNOLOGICAL METHODS vol. 110, no. 2, 1988, pages 169 - 178; O. KANAGAWA: 'Antibody-mediated activation of T cell clones as a method for screening hybridomas producing antibodies to the T cell receptor' see page 170, left column, paragraph 1 see page 171, left column, paragraph 2 -paragraph 3	1-38
X	---	39
Y	WORLD PATENTS INDEX LATEST Derwent Publications Ltd., London, GB; AN 88-080736 & JP,A,63 033 338 (AJINIMOTO) 13 February 1988 see abstract	1-38
X	---	39
	-/-	

### \* Special categories of cited documents: <sup>10</sup>

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
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\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, the combination being obvious to a person skilled in the art

\*A\* document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

29 NOVEMBER 1991

Date of Mailing of this International Search Report

23. 12. 91

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

C. TURMIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claims No.
Y	L.B.SCHOOK 'MONOCLONAL ANTIBODY PRODUCTION TECHNIQUES AND APPLICATIONS' 1987 , MARCEL DEKKER, INC , NEW YORK cited in the application Chapter 2; P.M.Allen: "Construction of murine T-T-Cell hybridomas"; pp25-35 see page 31, line 11 - line 42 ---	1-39
Y	L.B.SCHOOK 'MONOCLONAL ANTIBODY PRODUCTION TECHNIQUES AND APPLICATIONS' 1987 , MARCEL DEKKER, INC , NEW YORK cited in the application Chapter 10; B.B.Aggarwal et al.: "Production and Purification of Lymphokines and their monoclonal antibodies"; pp173-187 see page 182, line 20 - line 26 ---	1-39
A	S.M.KINGSMAN 'GENETIC ENGINEERING' 1988 , BLACKWELL SCIENTIFIC PUBLICATIONS , OXFORD Chapter 12; BIOTECHNOLOGY; pp414-456 see page 436 - page 444 ---	1-8
X	WORLD PATENTS INDEX LATEST Derwent Publications Ltd., London, GB; AN 90-243905 & JP,A,2 171 199 (TORAY) 2 July 1990 see abstract ---	39

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. CA 9100275  
SA 49943**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A-2485739	31-12-81	None	
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82